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14. ABSTRACT Lung cancer is the most common cause of cancer mortality in the United States. The majority of human lung cancers are adenocarcinoma histology and have been found to have somatic mutations in the genes that encode the EGFR/KRAS/BRAF signaling pathway. <i>KRAS</i> mutant adenocarcinomas of the lung are refractory to targeted agents and have been labeled "undruggable". The identification of additional molecular targets in <i>KRAS</i> mutant lung tumors that when inhibited result in a clinical response are needed. <i>Twist1</i> is a basic helix-loop-helix (bHLH) transcription factor that usually is undetectable in adult tissues, but has been shown to be overexpressed in some cancers. Interestingly, <i>Twist1</i> could also contribute to early tumorigenesis through inhibition of oncogene-induced senescence associated with <i>Kras</i> ^{G12D} . Therefore, Anti-TWIST1 agents could be useful against <i>KRAS</i> mutant lung cancer. We identified from a bioinformatics-chemical screen a candidate agent, harmine, that appears to target TWIST1-dependent pathways of tumor maintenance. Harmine decreased cell viability of lung cancer cells in a concentration dependent fashion that was correlated with increased markers of apoptosis and decreased TWIST1 protein levels <i>in vitro</i> . Harmine also decreased lung tumor growth rates in a genetically engineered mouse model of Twist1-induced lung adenocarcinoma that was correlated with increased markers of apoptosis and decreased TWIST1 protein levels <i>in vivo</i> .					
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Designing polyamide inhibitors of TWIST for pro-senescence therapy

PI – Phuoc T. Tran, MD, PhD

1. INTRODUCTION:

The proposed research program will develop novel inhibitors of TWIST1 for lung cancer. Non-small cell lung cancer (NSCLC) is the most common cancer diagnosed in the United States. It has been estimated that greater than 220,000 new cases of lung cancer will be diagnosed in the United States in 2014 and lung cancer will be responsible for the most common cause of cancer deaths (1). Recent efforts to classify distinct molecular subtypes of lung cancer have led to the novel findings that greater than 50% of adenocarcinoma of the lung can be separated into distinct oncogene driver classes (2).

The majority of human lung cancers are adenocarcinoma histology and have been found to have somatic mutations in the genes that encode the EGFR/KRAS/BRAF signaling pathway. These human data combined with animal models of this pathway suggest that EGFR/KRAS/BRAF forms a signaling axis of central importance to lung adenocarcinoma development (2-5). *KRAS* mutant adenocarcinomas of the lung are refractory to targeted agents and have been labeled “undruggable” (6). The identification of additional molecular targets in *KRAS* mutant lung tumors that when inhibited result in a clinical response are needed.

TWIST1 is a basic helix-loop-helix (bHLH) transcription factor that usually is undetectable in adult tissues, but has been shown to be overexpressed in some cancers. The high expression of *TWIST1* in cancers strongly correlates with highly invasive and metastatic cancers via the epithelial-mesenchymal transition (EMT) transdifferentiation program that allows epithelial cells to obtain a more migratory mesenchymal phenotype (7). Interestingly, *Twist1* could also contribute to early tumorigenesis through other mechanisms by its ability to inhibit oncogene-induced senescence (OIS) associated with activation of *Kras*^{G12D} and *EGFR2* oncogenes *in vitro* (8). OIS is a failsafe program that prevents normal cells from progressing towards malignancy following introduction of a mutant form of an oncogene such as *Kras*^{G12D} resulting in an irreversible cell cycle arrest (9). Therefore, TWIST1 may act both to induce malignancies early in tumorigenesis and promote cell dissemination later during tumor progression. However, the absence of a suitable animal model has made it difficult to date to determine how TWIST1 activation *in vivo* contributes to tumorigenesis and tumor progression. Recently, we published on a novel inducible *Kras*^{G12D}/*Twist1* lung cancer mouse model (10) and on human data that suggested TWIST1 as a promising therapeutic target for lung cancer treatment (11).

Pyrrole-Imidazole Polyamides (PIP) are a class of cell permeable programmable small-molecule heterocyclic amino acid oligomers that can be designed to bind pre-determined DNA sequences with high affinity and specificity. These small molecules can inhibit DNA binding of a broad range of transcription factors, localize to the cell nucleus, bind to chromatin, and down-regulate gene expression *in vitro* (12). PIP have been used to antagonize the androgen receptor, glucocorticoid receptor and hypoxia inducible factor-1 *in vitro*. We hypothesized that a PIP could be synthesized to inhibit TWIST1. We hoped our one-year proposal would allow us to generate the critical initial data to support more extensive, mechanistic studies in the future.

The original specific aims are below:

Specific Aim#1. Design and synthesize a TWIST1-inhibitory specific Pyrrole-Imidazole Polyamides (PIP).

Rationale: Data from human cell lines and transgenic mouse models suggest TWIST1 as target for *KRAS* mutant lung cancers. PIPs are cell permeable programmable small-molecule heterocyclic amino acid oligomers that can be designed to bind pre-determined DNA sequences with high affinity and specificity and inhibit transcription factors such as TWIST1.

Study Design: Synthesize PIPs by established solid phase synthesis protocols to target the TWIST1 DNA binding motif, E-box, found in the *CDH1* promoter. After synthesis and purification, characterize PIPs by analytical physical methods and then using *in vitro* quantitative DNase I footprint assays and gel shift competition experiments.

Specific Aim#2. Determine efficacy of Twist1-inhibitory PIPs for abrogation of TWIST1-dependent functions *in vitro* and *in vivo*

Rationale: TWIST1-inhibitory PIPs synthesized need to be validated biologically for TWIST1 inhibition and OIS activation *in vitro*. PIPs validate from our cell based screen will be further tested for tumor responses and

OIS activation via Twist1 inhibition *in vivo* using transgenic autochthonous *Kras*^{G12D}/*Twist1*-induced lung tumor models.

Study Design: Test the top candidate anti-TWIST1 agents using human *KRAS* mutant lung cancer cell lines for cellular viability, clonogenic survival and for activation of OIS by inhibiting TWIST1 using cell based and molecular assays. The most promising candidate anti-TWIST1 agents from our *in vitro* screen on human *KRAS* mutant lung cancer cells will be validated *in vivo* using a transgenic autochthonous *Kras*^{G12D}/*Twist1*-induced lung tumor model. Following treatment of tumor moribund mice with candidate PIPs from our cell based screen, we anticipate that a subset of these anti-TWIST1 agents will show a clinical effect on tumor growth and activation of OIS via inhibition of Twist1 *in vivo*.

2. KEY WORDS:

Pyrrole-Imidazole Polyamides; TWIST1; KRAS; non-small cell lung cancer (NSCLC); senescence.

3. OVERALL PROJECT SUMMARY:

Progress is listed in relation to each specific task in the “Statement of Work” and highlighted by *italics* for the past year of this one year proposal.

Task#1 - Design and synthesize a TWIST1-inhibitory specific Pyrrole-Imidazole Polyamides (PIP) (months 1-6).

1a. Designing and synthesizing PIPs (months 0-2).

The general chemical synthesis scheme for [ImImPyPya-aminoy-PyPyPyPy] polyamide as indicated in our DoD proposal was obtained from the original article published by the Dervan group (13, 14). Prior to synthesis, we initiated conversation with one of the author's on these manuscripts to obtain key information that could help us with the synthesis scheme and obtain high yields. This is when we realized that the established synthetic scheme for [ImImPyPya-aminoy-PyPyPyPy] polyamide has not been reported. The synthesis involves multiple steps using the protocols mentioned in the above references. Also according to the published protocol, it seems likely that a significant amount of polyamide may be lost during purification, depending on the precise column conditions and loading. Finally and most importantly, we also realized that we needed access to specialized instrument such as microwave (Biotage Initiator Eight), Analytical UPLC and/or Parr hydrogenation apparatus to complete the synthesis. Given the lack of access to these very specialized instrumentation and ill-defined chemical synthesis scheme our ability to successfully synthesize the compound was severely compromised.

1b. Characterization of PIPs by HPLC, UV-visible spectroscopy, and matrix-assisted laser desorption ionization/time of flight mass spectrometry (months 1-3).

1c. Perform DNase I footprinting experiments (months 2-4).

1d. Perform gel shift experiments (months 3-5).

Due to the difficulties listed above we could not proceed with any of these downstream tasks as they are completely dependent on the steps above.

Task#2 - Determine efficacy of Twist1-inhibitory PIPs for abrogation of TWIST1-dependent functions *in vitro* and *in vivo* (months 1-12).

2a. IACUC and other regulatory approval process for animal work (months 0-2).

2b. Mating mice for experiments (months 2-12).

As above in Task #1b-d we were unable to proceed with a PIP designed compounds as originally proposed. However, we were able to proceed using a different compound that we retrieved from a bioinformatic-chemical screen called harmine. The following work will describe studies performed using this compound in place of PIPs. We applied for and were approved by the IACUC for animal work using PIPs and harmine. The appropriate mice were mated to produce CCSP-rtTA/tetO-Kras^{G12D}/tetO-Twist1(CRT) mice to use for our *in vivo* validation experiments (Task #2e).

2c. In vitro validation of PIPs (months 3-12).

*We demonstrated that harmine decreased cellular viability in a range of NSCLC cell lines in a concentration dependent fashion (**Appendix Fig 1A**) that was correlated with increased markers of apoptosis (**Appendix Fig 1B-C**) and decreased TWIST1 protein levels (**Appendix Fig 2**)*

2d. Performing toxicity, pharmacokinetic and pharmacodynamics studies (months 4-6).

These are on-going with harmine.

2e. Performing tumor studies (months 6-12).

*We demonstrated that harmine decreased tumor growth rate in our CRT mouse model of lung adenocarcinoma when given at 10 mg/kg per intraperitoneal injection for 5 days a week for three straight weeks compared to vehicle control treated CRT mice (**Appendix Fig 3**).*

2f. Collecting tissues from experiments (months 7-12)

2g. Performing experiments on tissues from mice (months 6-12).

2h. Analyzing results of experiments on tissues from mice (months 7-12).

*We have collected lung tumor samples from vehicle and harmine treated CRT mice. The preliminary analysis of these lung tumor tissues suggest that TWIST1 levels are decreased by TWIST1 Western blotting (data not shown). We do not see any effects of harmine on proliferation by Ki-67 immunohistochemistry (IHC) (**Appendix Fig 4A**) suggesting that activation of senescence is not likely, but we do see increased apoptosis in harmine treated CRT mouse lung tumors by cleaved caspase-3 (CC3) IHC (**Appendix Fig 4B**).*

4. KEY RESEARCH ACCOMPLISHMENTS:

- *Identification from a bioinformatics-chemical screen of a candidate agent, harmine, that appears to target TWIST1-dependent pathways of tumor maintenance.*
- *Initial validation that harmine decreases cell viability of NSCLC cells in a concentration dependent fashion that was correlated with increased markers of apoptosis and decreased TWIST1 protein levels in vitro.*
- *Initial validation that harmine decreases lung tumor growth rates in a genetically engineered mouse model of lung adenocarcinoma that was correlated with increased markers of apoptosis and decreased TWIST1 protein levels in vivo.*

5. CONCLUSION:

During this one year of support we have not been able to strictly adhere to the timeline of our “Statement of Work”; namely because of technical issues with Task#1a (as detailed above in the section **3**).

OVERALL PROJECT SUMMARY. As a result of these technical issues, this has precluded us from pursuing the remaining Tasks #1b- d and #2 in full with a PIP synthesized anti-TWIST1 agent as we proposed originally. However, we have salvaged the core of our proposal by examining another agent, harmine, isolated from a bioinformatic-chemical screen. We have been very successful with using harmine to fulfill many of the remaining tasks and have developed strong data on the efficacy of harmine to target TWIST1 in a post-translational manner for degradation and in turn affect TWIST1-dependent tumor maintenance pathways in NSCLC cells *in vitro* and lung tumors *in vivo*. Our future plans include further validation of our promising findings with harmine and a more detailed mechanistic understanding of how harmine results in post-translational downregulation of TWIST1.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:

a. List of manuscripts submitted for publication during the period covered by this report resulting from this project.

1. None. We have a manuscript in preparation describing the harmine studies above.

- b. List presentations made during the last year (international, national, local societies, military meetings, etc.).
1. Stanford University Medical Center Radiation Oncology Visiting Professor (October 28, 2013). "Structure-functions studies of the TWIST1 oncoprotein in lung and prostate cancer".
 2. UC San Diego, Moores Cancer Center Radiation Oncology Faculty Candidate (April 18, 2014). "Structure-functions studies of the TWIST1 oncoprotein in lung and prostate cancer".
 3. Sidney Kimmel Comprehensive Cancer Center (SKCCC) at Johns Hopkins University Chemical Therapeutics Faculty Meeting Seminar (September 8, 2014). "Bioinformatic-chemical screen for inhibitors of Twist1".

7. INVENTIONS, PATENTS AND LICENSES:

None.

8. REPORTABLE OUTCOMES:

- Harmine as a novel anti-TWIST1 agent that operates by post-translational downregulation *in vitro* and *in vivo*.

9. OTHER ACHIEVEMENTS:

- No degrees were obtained that are supported by this award.
- We did not develop any cell lines or serum repositories, but tissues from our mice were banked for further analysis as described above in the section **3. OVERALL PROJECT SUMMARY**.
- No additional funding has been applied for based on this work yet, but we have plans for this coming year to apply for additional funding examining the role of harmine and harmine derivatives as anti-TWIST1 agents in a variety of other cancers.
- No employment or research opportunities applied for and/or received based on experience/training supported by this award.
- No degrees were obtained that are supported by this award.
- No informatics databases were constructed as above in the section **3. OVERALL PROJECT SUMMARY**.

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11. APPENDIX:

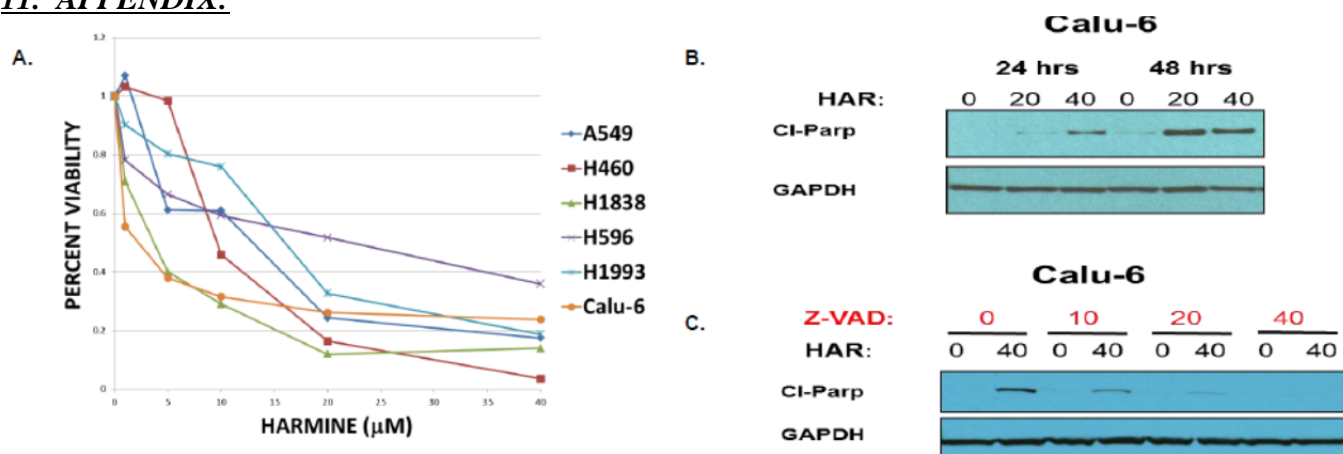


Fig 1. Harmine inhibits growth through the induction of apoptosis in NSCLC cell lines. (A) Representative MTS assays demonstrating growth inhibition of in the indicated NSCLC cells in a dose and time dependent manner following harmine treatment at the indicated doses and timepoints. (B) Western blot demonstrating a marked increase in PARP cleavage following harmine treatment at the indicated doses and timepoints in the *KRAS* mutant NSCLC cell line, Calu-6 (C) Western blot showing harmine induced PARP cleavage can be prevented by co-treatment with Z-VAD, a pan caspase inhibitor. Cells were co-treated with harmine and Z-VAD for 24 hrs. at the indicated mM concentrations (DMSO was used as control) and then harvested for Western blot analysis.

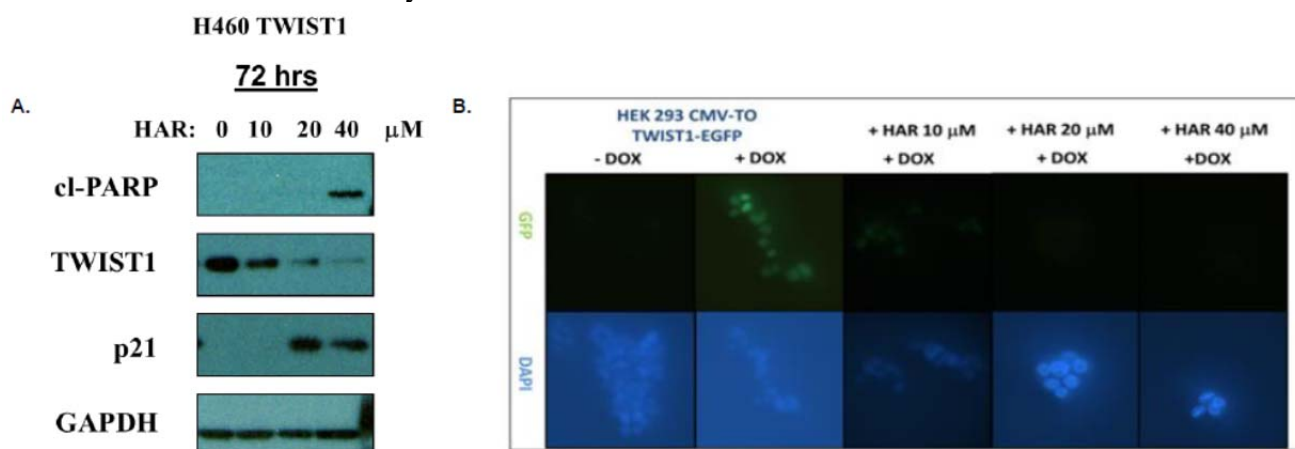


Fig 2. Harmine treatment leads to decreased TWIST1 protein levels. (A) Western blot demonstrating reduction of exogenous TWIST1 protein expression as well as induction of p21 and cleaved Parp in H460 TWIST1 overexpressing cells 72 hrs. after treatment with the indicated doses of harmine. (B) Fluorescence microscopy (20X) demonstrating nuclear localization of TWIST1-EGFP (green). DAPI stain (blue) was used to stain the nucleus after addition of 500 ng/ml doxycycline and subsequent decrease in TWIST1 expression after co-treatment with increasing doses of harmine.

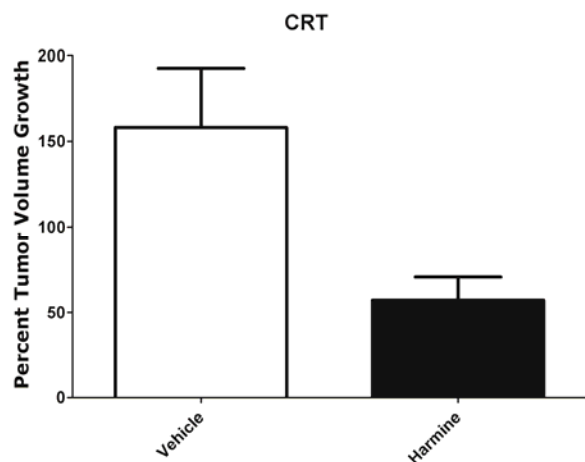


Fig 3. Harmine treatment slows tumor growth *in vivo*. Treatment of lung tumor moribund CCSP-rtTA/tetO-KrasG12D/tetO-Twist1 (CRT) mice with 10mg/ml of harmine IP or vehicle control for three cycles of Mon-Fri daily treatment. Tumor volumes were quantified at the end of the experiment by microCT and normalized to pre-treatment starting tumor volumes ($p < 0.5$ by Mann-Whitney t -test).

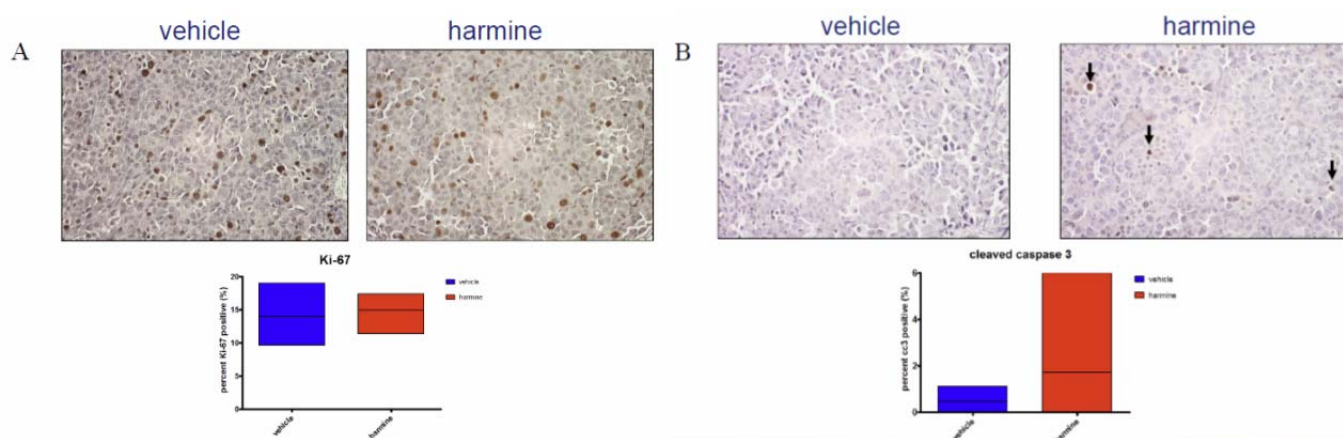


Fig 4. Harmine treatment causes more lung tumor cell apoptosis *in vivo*. Treatment of lung tumor moribund CCSP-rtTA/tetO-KrasG12D/tetO-Twist1 (CRT) mice as above in Fig 3. Lung tumors were harvested and analyzed by IHC for (A) proliferation by Ki-67 and (B) apoptosis with cleaved caspase 3.

Complete Bibliography:

See section **#6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS.**

Personnel:

1. Phuoc T. Tran, MD, PhD - PI
2. Saritha Tantravedi, PhD – Postdoctoral chemist
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